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AWARD NUMBER DAMD17-94-J-4431

TITLE: Immunotherapy of Congenital SIV Infection

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REPORT DATE: October 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1998	Annual (30 Sep 97 - 29 Sep 98)

4. TITLE AND SUBTITLE
Immunotherapy of Congenital SIV Infection

5. FUNDING NUMBERS
DAMD17-94-J-4431

6. AUTHOR(S)
Ruth M. Rupprecht, M.D.,Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Dana-Farber Cancer Institute
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

During the no-cost extension, the animals enrolled in the DNA vaccine study received 2 protein boosts with recombinant gp160 derived from HIVIIIB. We have enrolled another control group of 4 age-matched rhesus macaques that received only the 2 gp160 boosts. At the time of challenge, several animals had developed high levels of neutralizing antibodies. A few weeks ago, all animals were challenged with 10 animal infectious doses of the homologous SHIV-vpu$^+$, a chimeric strain that contains the HIVIIIB *env* gene. While early results indicate that some animals may have been protected against virus challenge, longer follow up will be required to determine the outcome.

14. SUBJECT TERMS	15. NUMBER OF PAGES
DNA vaccines/SHIV virus/rhesus macaques	22
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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/Ruth Reynolds/ 11/4/98

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INTRODUCTION

As mentioned in the report earlier this year, the overall goal of this project is to develop DNA vaccine strategies to treat and/or prevent primate lentivirus infection in infant rhesus macaques. As mentioned previously, the original Specific Aims had been readjusted, and we had entered into a new affiliation with the Yerkes Regional Primate Research Center (Atlanta, GA). This switch was necessitated by our previous difficulties in obtaining sufficient numbers of retrovirus-free pregnant rhesus monkeys. The YRPRC maintains a large, retrovirus-free rhesus monkey breeding colony. With our highly sensitive polymerase chain reaction (PCR) assays (Liska et al., 1997a, b), we have never detected evidence of infection with either simian retrovirus type D (SRV/D) or simian T-lymphotropic virus type I (STLV-I). Approximately 2 years ago, 28 newborn rhesus macaques were entered into a DNA vaccine trial in groups of 4. This experiment is being conducted in parallel to a similar trial carried out in adult macaques Dr. Harriet Robinson, a collaborator on our current study. This experimental design allowed us to test whether DNA vaccines exhibit age-related differences in immunogenicity, as discussed previously. Should the vaccines be safe as well as immunogenic in neonatal primates, important information can be generated to apply this new technology worldwide for childhood vaccination.

As mentioned in the previous report, the initial inoculations with naked DNA expression vectors yielding disappointing results. As a consequence, the protocols were modified, and 2 boosts with recombinant gp160 derived from HIVIIIB were added. Animals in groups that had not been vaccinated with DNA plasmids encoding the *env* gene were an additional 2 DNA vaccine boosts. Exactly 2 weeks after the last boost, the animals were challenged intravenously with 10 50% animal infectious doses (AID_{50}) of the homogolous SHIV-vpu^+.

This current report summarizes work conducted during the past 6 months, including the early data obtained after the virus boosts.

Publications since the report of April, 1998:

Rasmussen RA, Sharma PL, Hu Y, Ruprecht RM. Vaccine protection by subinfectious doses of a live murine leukemia virus. *Leukemia*, in press.

MATERIALS AND METHODS

This section will focus on methodologies used since April, 1998.

1. DNA vaccination of infant rhesus monkeys. Groups of 4 infant rhesus macaques enrolled in the vaccine study as outlined previously had received DNA vaccines at 0, 4, 6, and 11 months. Blood samples have been collected at birth, at 2 weeks, 6 weeks, 5½ months, 6½ months, 10½ months and 12 months. Antibody levels are being followed by ELISA, Western blot analysis and neutralization using autologous virus.

2. Administration of gp160 boosts. Recombinant gp160, prepared from the HIV-1 strain LAI, which is almost identical to HIVIIIB, was kindly provided by Dr. Shiu-Lok Hu (University of Washington, Seattle). Each animal received 100 μg of gp160 mixed 1:1 (volume:volume) with incomplete Freund's adjuvant (ICFA, Sigma). For 20 animals, 1.9 ml of gp160 (1.1 mg/ml in 2 x phosphate-buffered saline) were mixed with 1.9 ml of ICFA. The ICFA was drawn up using an 18G needle. The gp160 was drawn up using a Pipetman and placed into the syringe bore through the plastic Pipetman tip. The mixture was then emulsified by drawing back and forth 100x using a 3-way stopcock system, as follows: The antigen was emulsified into the ICFA using glass syringes. The volume of the glass syringes was chosen to be able to hold the total volume of both the antigen and the adjuvant in 1 syringe. The adjuvant was first drawn up into one syringe, while the antigen was drawn up into the other syringe. Both syringes were attached through the 3-way stopcock, making sure that no air was within the stopcock or in the syringes. With the first press of the syringe barrel, the adjuvant was put into the syringe containing the antigen. Then the mixture was drawn back and forth until a fine emulsion was obtained which eventually turned white. No separate oil droplets were noticed at the time the mixture was ready for injection into the animals.

   The recombinant gp160 was inoculated intramuscularly (i.m.) at a total dose of 100 μg per animal per dose.

3. Intravenous challenge of the vaccinated macaques with SHIV-vpu$^+$. Exactly 2 weeks after the last boost with gp160 (groups 1, 2, 6, 7, and 8; Table 1) or with the appropriate DNA expression vectors used for prior inoculations (groups 3, 4, and 5; Table 1), the animals were challenged i.v. with 10 AID_{50} of SHIV-vpu$^+$, the homologous virus strain. The stock had been titrated previously i.v. in adult macaques and was kindly provided by Dr. Yichen Lu (Virus Research Institute, Cambridge, MA).

4. Virologic assays. Blood samples were collected aseptically in sterile preservative-free heparin anticoagulant. Plasma and peripheral blood mononuclear cells (PBMC) were separated by Ficoll density gradient

centrifugation. PBMC were cultured in the presence of CEMx174 cells as described (Baba et al., 1994). After 21 days, cultures supernatants were collected for determination of p27 Gag antigen levels using a commercial kit (Coulter, Miami, FL).

5. Western blot analysis of specific anti-SHIV immune responses. Commercially available HIV-1 Western blot strips were used to measure anti-HIV-1 Env responses. Because the Gag antigens are well conserved between HIV-1 and SIV, we expect to find anti-Gag responses by this Western blot analysis as well; however, the serum samples were also tested for anti-SIV responses also by commercially available HIV-2 strips, which we have used extensively for our previous SIV work (Baba et al., 1994; 1995).

6. SIV-specific CTL responses in macaques. CTL assays in the rhesus macaque system were established by evaluating adult macaques infected with SIVmac251. All CTL assays or samples from the experimental animals include a positive control from an SIVmac251-infected adult macaque known to be CTL positive. Numerous aliquots of effector cells from this control animal have been cryopreserved.

6a. Recombinant virus vectors. Recombinant vaccinia viruses containing wild type vaccinia or *gag*, *pol*, or *nef* genes of SIV have been obtained from Dr. Panicali (Therion Biologics Corp., Cambridge, MA). The appropriate recombinant vaccinia viruses to measure CTL activity in SHIV-DNA-vaccinated animals have been obtained also.

6b. Preparation and labeling of B-lymphoblastic cell lines (B-LCL). Peripheral blood samples were collected in sterile, preservative-free heparin and shipped to the Dana-Farber Cancer Institute on ice. PBMC were separated on Ficoll-hypaque gradients and infected with cell-free herpesvirus *papio*. Autologous B-LCL target cells were labeled with ^{51}chromium and infected overnight with wild-type vaccinia or recombinant vaccinia vectors containing either a single SIV *gag/pol/env* gene insert, individual SIV *gag-pol*, SIV *nef*, or HIVIIIB *env* gene inserts.

6c. CTL effector cell cultures. PBMC from the experimental monkeys were obtained by Ficoll-hypaque gradient centrifugation of blood. PBMC were cultured in RPMI-1640 media supplemented with 15% FCS, penicillin, streptomycin, and L-glutamine. Mitogen-stimulated PBMC effector cells were cultured in medium containing a 5 μg/ml Concanavalin A (ConA) for 3 days, washed and cultured for 4 days in medium containing 10% human IL-2. Other cultures were stimulated for 2 days with autologous paraformaldehyde-fixed B-LCL infected with a vaccinia vector containing an SIV *gag/pol/env* or an HIVIIIB *env* gene construct, and then cultured for 5 days in medium containing 10% human IL-2.

6d. SIV-specific cytotoxic CTL assays. SIV-specific CTL activity was determined using various effector-to-target cell (E:T) ratios in a standard 5 h ^{51}Cr release assay. The percentage of specific cytotoxicity was determined from the formula: 100 (experimental release - spontaneous release)/(maximum release - spontaneous release).

6e. CTL assays in the presence of cold targets. Because CTL assays with effector cells of some vaccinated infants revealed high backgrounds, the assays were repeated in the presence of unlabeled ("cold") target cells as follows. The cold-hot target cell ratio was 10:1. Cold targets were prepared from autologous B-LCL by incubation overnight with wt-vaccinia construct in the absence of ^{51}Cr.

RESULTS

As mentioned in the previous sections, this report covers the period from April, 1998 to October, 1998. The project is on a continued no-cost extension, which was necessitated by the unexpectedly poor immunogenicity of DNA expression vectors when used alone.

1. Boosting of experimental macaques after priming with DNA expression vectors.

Our overall experimental plan was amended to include boosts as outlined in Table 1. All animals tolerated the boosts with recombinant gp160 well. No acute reactions were noted. Likewise, the additional 2 inoculations with DNA (groups 3, 4, and 5) were well tolerated.

2. Recombinant gp160 induced strong neutralizing antibody responses.

At the end of the scheduled inoculations with DNA expression vectors, none of the animals had developed measurable neutralizing antibodies, as discussed in the last report. In some of the animals, antibodies against the HIV envelope glycoproteins had developed, as measured by ELISA. The complete lack of neutralizing antibodies after the repeated DNA vaccination had led us to add the boosts with recombinant gp160.

Two weeks after the second boost, on the day of virus challenge, neutralizing antibodies were detected in the majority of the animals given recombinant gp160 (Table 2). In group 1, all animals were positive, whereas 2 out of the 4 animals in group 2 had neutralizing antibody levels ranging in the 40s. As expected, groups 3, 4, and 5, which had never been exposed to DNA expression vectors encoding *env* and had not been boosted with recombinant gp160, had no measurable neutralizing antibodies. In group 6, which consists now of 3 animals only, 1 animal had high levels of neutralizing antibodies. The highest neutralizing antibody level ever seen to my recollection in any DNA vaccine study was seen in animal RFw5 in group 7, which had a neutralizing antibody level of 2,010. Only 1 animal in this group remained negative for neutralizing antibodies. Most interestingly, 3 out of the 4 naive animals given 2 inoculations with recombinant gp160 developed high levels of neutralizing antibodies. These animals had not been given any prior DNA vaccines. Lastly, the naive controls which were left untreated were negative.

3. Early results of intravenous challenge with homogolous SHIV-vpu+.

In mid-September, 1998, all 9 groups of animals were challenged i.v. with 10 AID_{50} of the homogolous SHIV-vpu+. Thus far, we have completed the virus co-cultivations of PBMC obtained 2 weeks post-challenge. Culture supernatants were analyzed for the level of SIV p27 Gag antigen after 3 weeks of co-cultivation. The results are expressed as the number of infectious cells per 10^6 PBMC (Table 2). At this early time point, all control animals that were given repeated inoculations of vector backbone

only (group 5) as well as the 4 naive animals (group 9) were virus-isolation positive. Virus was isolated also from all animals in groups 3 and 4 which had been vaccinated repeatedly with DNA expression vectors expressing *gag*, *pol*, and *nef* (Table 2). In contrast, no infectious cells were found in 1 of 3 animals of group 6, in 1 of 4 animals of group 7, and 2 of 4 animals of group 8 (Table 2).

Additional co-cultivation results are pending from blood samples collected at weeks 4 and 6 post-challenge. We plan to monitor all animals for the number of infectious PBMC for several more weeks. While we recognize that lack of infection noted in 4 of the vaccinated infants at the 2-week time point post-challenge may not hold upon further study, it is interesting to note that each of these protected animals had high titers of neutralizing antibodies that ranged from 138 to 2,010.

Western blot analyses using HIV-2 strips were performed on blood samples collected 4 weeks post-challenge. Among the control animals in groups 5 and 9, 5 out of 8 animals were positive for anti-Gag antibodies at this time point. Interestingly, among animals from groups 1, 2, 6, 7, and 8, which had been given recombinant gp160 boosts, only 2 of 19 had anti-Gag antibody responses by Western blot at 4 weeks. It will be interesting to follow these animals for longer periods of time to see whether seroconversion will be significantly delayed or absent in these vaccinees.

At the time of this writing, results from other studies are pending (DNA PCR, RT-PCR, ELISA, and CTL activity at the time of challenge).

4. Health status of the vaccinated macaque infants.

In the previous report, we had described diarrheal illnesses that had developed in some of the vaccinated infants. A review of the records has revealed that in most cases, the diarrhea was due to an infectious cause. *Campylobacter* was isolated from separate animals, including also from adult animals with diarrhea. It should be noted that these adult animals are enrolled in different studies. Consequently, we conclude that the diarrhea is most likely due to nosocomial infections due to pathogens endemic in the colony. At the present time, it seems unlikely that the DNA vaccination was the cause of the diarrheal illnesses.

One animal of group 6 had to be euthanized due to the severity of its diarrheal illness that failed to respond to antibiotic treatments. This animal, REw5, was euthanized on 06/17/98. The necropsy revealed the following: mild emaciation, severe dehydration, calcified nodule at the right A-V valve, lymphadenopathy involving mesenteric and peri-aortic lymph nodes, mild spondylosis of the lumbar spine, chronic pleuritis, campylobacteriosis of the colon.

DISCUSSION AND FUTURE PLANS

During the remaining no-cost extension, we plan to complete the follow up of the vaccinated infants. Early results indicate that possibly 50% of the infants given gp160 only, in the absence of prior priming with DNA vaccines, may be protected against homogolous virus challenge. Preliminary findings indicate that high levels of neutralizing antibodies may correlate with protection. Before drawing firm conclusion on these latter 2 statements, the animals need to be followed for longer periods of time, as we discussed previously. Furthermore, 2 animals vaccinated with DNA expression vectors via gene gun administration, 1 with and 1 without adjuvant IL-12 followed by 2 boosts with recombinant gp160, have remained virus-isolation negative at the early time point.

We should also point out that the number of infectious cells per 10^6 PBMC was low in several other animals with numbers ranging between 1 and 4 infectious cells per 10^6 PBMC. None of these animals have seroconverted to the Gag antigens at 1 month post-challenge by Western blot. Future studies may reveal that the virus loads in some of these animals may remain significantly lower as compared to the non-vaccinated control animals.

If co-cultivation, DNA PCR, RT-PCR, ELISA, and Western blot analyses fail to document persistent infection in the 4 animals that have remained virus-isolation negative, or transient infection in some of the animals with 1 to 4 infectious cells 10^6 PBMC, we plan to perform lymph node biopsies. Lymph node specimens will be analyzed by co-cultivation and DNA PCR for the presence of virus.

Animals without persistent infection and without seroconversion after challenge with SHIV-vpu$^+$performed in September, 1998, will be challenged with a higher dose of homologous virus. If they still remain protected, a heterologous SHIV challenge with SHIV-89.6p will be conducted. This latter virus encodes the *env* gene of a primary HIV-1 isolate and is highly pathogenic in rhesus macaques.

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Table 1. DNA Vaccine Experiment in Neonatal Macaques with IL-12 as Adjuvant: Boosting Protocol

Group #	n	DNA	IL-12	DNA Vaccine Delivery	gp160 boost	DNA boost
1	4	5 DNAs	-	ID	IM	-
2	4	5 DNAs	+	ID	IM	-
3	4		-	ID	-	*gag-pol/nef* DNAs ID
4	4		+	ID	-	*gag-pol/nef* DNAs ID
5	4	control DNA	-	ID	-	control DNA ID
6	4	5 DNAs	-	gun	IM	-
7	4	5 DNAs	+	gun	IM	-
8	4	-	-	gun	IM	-
9*	4	-	-	-	-	-

ID = intradermal

IM = intramuscular

* = animals in group 9 are naive controls to assess the infectivity of the virus stock upon challenge

Legend.

Because of the unexpected low immunogenicity of the DNA vaccines given as outlined in Table 1, animals primed with *env*-expressing DNA vectors (Groups 1, 2, 6, and 7) will be boosted with purified recombinant gp120 prepared from HIV-1 strain LAI (kindly provided by Dr. Shiu-Lok Hu).

Table 2. Infectious cells per 10^6 PBMC 2 weeks post-challenge and neutralizing antibody titers at the time of challenge

Week 2

Animal #	Group #	DNA Vaccination	# infectious cells per 10^6 PBMC	# PBMCs Rqd for SHIV isolation	Serum dilution with neutralizing Abs
REu-5	1	5 DNAs	4	250,000	89
RJu-5	1		64	15,625	45
RIv-5	1		1	1,000,000	146
RUv-5	1		16	62,500	49
RDv-5	2	5 DNAs + IL-12	256	3,906	<20
RTV-5	2		64	15,625	47
RYv-5	2		64	15,625	<20
RMw-5	2		4	250,000	41
RWt-5	3	gag/pol/nef	1024	977	<20
ROu-5	3		1024	977	<20
RBv-5	3		64	15,625	<20
ROv-5	3		64	15,625	<20
RTu-5	4	gag/pol/nef + IL-12	256	3,906	<20
RJv-5	4		256	3,906	<20
RSv-5	4		1024	977	<20
RCw-5	4		16	62,500	<20
RIu-5	5	control DNA	1024	977	<20
RWu-5	5		1024	977	<20
RZu-5	5		16	62,500	<20
RVv-5	5		256	3,906	<20
RAw-5	6	genegun (5)	1024	977	<20
RBw-5	6		64	15,625	<20
RDw-5	6		0	> 10^6	138
RFw-5	7	genegun + IL-12	0	> 10^6	2010
RGw-5	7		1	1,000,000	115
RIw-5	7		16	62,500	51
RJw-5	7		64	15,625	<20
RGt-5	8	gp160 alone	16	62,500	410
RDt-5	8		0	> 10^6	145
RNs-5	8		1024	977	<20
RTs-5	8		0	> 10^6	403
RNo-5	9	Challenge only	1024	977	<20
ROs-5	9		4	250,000	<20
RSp-5	9		1	1,000,000	<20
Rko-5	9		256	3,906	<20

LIST OF APPENDIX ITEMS

Rasmussen RA, Sharma PL, Hu Y, Ruprecht RM. Vaccine protection by subinfectious doses of a live murine leukemia virus. *Leukemia*, in press.

Vaccine Protection by Subinfectious Doses of a Live Murine Leukemia Virus. Robert A. Rasmussen, Prem L. Sharma, Yuwen Hu, and Ruth M. Ruprecht

Laboratory of Viral Pathogenesis, Dana-Farber Cancer Institute and Department of Medicine, Harvard Medical School, Boston MA 02115

We have postulated that following retrovirus infection, disease will only develop if a certain threshold of virus load is surpassed in a given host. No disease will occur if overall viral burden is maintained below such a threshold, even if the retrovirus encodes all genes necessary for virulence. Furthermore, we hypothesize that host cellular defense mechanisms are key determinants for this threshold effect.

Evidence for cell-mediated immune defenses as a mechanism for clearance of retrovirus infection was demonstrated in our previous studies using the Rauscher murine leukemia virus (RLV) model[1,2]. Protective immunity could be generated by inoculating mice with a potentially lethal dose of RLV; viral replication was blocked by combination antiretroviral drug therapy (AZT and interferon-α). Mice were inoculated with high-dose virus and 4 hrs later, drug treatment was started and continued for 3 weeks. After this time, animals showed no evidence of infection and were rechallenged with a fully infectious dose of RLV. More than 95% of the animals developed neither viremia nor disease[1]. Adoptive transfer experiments indicated that protective immunity was mainly cellular and required both $CD4^+$ and $CD8^+$ T lymphocytes[2]. DNA PCR analysis revealed that virus replicated to low levels despite antiviral therapy. Eventual provirus clearance coincided with the emergence of RLV-specific CTL responses, mediated predominantly by $CD8^+$ T cells.

To test whether host cell immunity alone could protect against low doses of live retrovirus, we titrated an RLV stock in normal, immunocompetent BALB/c mice and in isogenic, T-cell deficient nude mice (Table 1). The apparent infectivity was 10-fold greater in the T-cell deficient mice, demonstrating that cell-mediated immunity is required to achieve viral clearance after low-dose inoculation. Normal mice exposed to subinfectious doses, i.e., mice without persistent viremia after exposure to low doses of live virus, were rechallenged with fully infectious doses to measure whether low-dose inoculation with live RLV could induce protective immunity (Table 2). Overall, 30% of the mice that definitely received live RLV in the initial low-dose inocula remained virus free after rechallenge with higher doses of RLV.

Low-dose infection can induce immune protection against certain other pathogens. Empirically, low-dose vaccinations were carried out through the practice of variolation in the 18th century in attempts to prevent smallpox[3]. More recently, it was shown that normally susceptible mice inoculated with low-dose *Leishmania major* are resistant to subsequent, normally pathogenic challenge doses[4]. Protection is associated with cell-mediated immunity, while *L. major*-specific antibody responses are diminished in a manner similarly described in earlier studies as "low-dose paralysis" [5]. Low-dose exposure to hepatitis B virus or lymphocytic choriomeningitis virus can also evoke protection against disease [6,7]. In these cases, the protective immune response is apparently but one element of a complex, dynamic balance between virus and host immunity which can potentially include immunopathologic consequences at higher virus doses[8].

Our data are consistent with a general hypothesis, outlined schematically in Figure 1, which describes potential short-term and long-term outcomes following retrovirus exposure. Central to this hypothesis is the idea that disease will occur only if viral loads exceed a certain disease threshold level. If viral load is maintained below this level, no disease will occur, even if the virus is fully replication competent and pathogenic. We also postulate the existence of both a vaccine threshold – a level of viral load which must be attained to induce protective immunity in the host animal, and a virus detection threshold, below which it cannot be determined whether a host animal harbors virus. In our mouse model, animals inoculated with high-dose RLV and left untreated are represented by Pathway (1), which shows viral load exceeding the disease threshold. More than 95% of mice given high-dose RLV inoculation plus drug treatment, and 30% of mice exposed to low-dose, live virus are represented by Pathway (2). Here, a transient level of viral load was induced, high enough to surpass a putative vaccine threshold level and generate a protective cellular immune response, yet kept below disease threshold levels. The remaining 70% of low-dose, RLV-inoculated animals that cleared virus but did not resist challenge are represented by Pathway (3). In these animals, transient viral load was not sufficient to generate protective immunity.

The results of this study clearly demonstrate that inoculation with a live, pathogenic retrovirus, at doses that are insufficient to establish persistent viremia, can induce protective immunity against subsequent challenge with a potentially lethal dose of virus.

This work was supported by grant #DAMD-17-94-J-4431 from the U.S. Army.

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Figure 1. Threshold Hypothesis of Retrovirus Infection. Based on this model, 3 threshold levels of virus load exist; the lowest level which can be assigned with any certainty is the virus detection threshold. In any system, viral loads below this level are unknown quantities. After virus exposure, 3 potential pathways are described: (1) unchecked retrovirus replication results in a viral load exceeding the disease threshold; (2) viral load is kept below disease threshold levels through drug intervention (open arrow, Rx), replication defects, or by natural host factors, yet transient levels of infection are high enough to generate protective immunity; or (3) virus is detected yet virus loads are too low to initiate a protective immune response. After infection, a state of viral latency may exist with periodic virus production which is suppressed by immune surveillance mechanisms (4). Reactivation of virus production, in the absence of adequate immune protection, will result in disease progression (5)

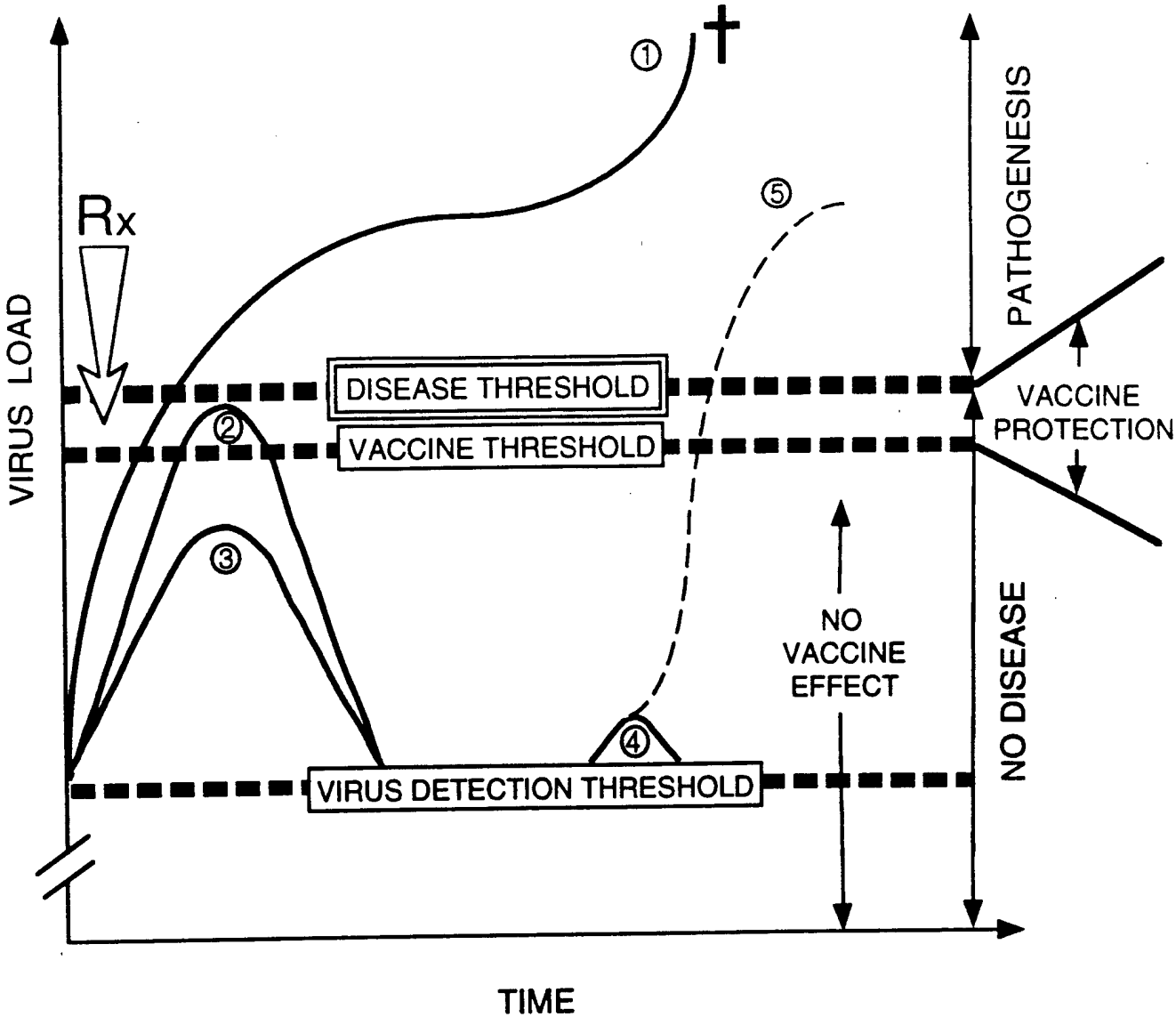
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Figure 1

Table 1. Infectivity of an RLV Stock in Immunocompetent and T Cell Deficient Mice

Virus Dose (AID_{50})	Percent of Mice with Persistent Viremia (No. Mice Viremic/No. Mice Inoculated)	
	Normal BALB/c mice	BALB/c *nu/nu*
20	100 (20/20)	100 (4/4)
2	70 (14/20)	100 (4/4)
0.4	30 (6/20)	100 (4/4)
0.2	5 (1/20)	75 (3/4)
0.04	5 (1/20)	25 (1/4)

Legend Table 1. Normal BALB/c or nude, athymic BALB/c *nu/nu* mice were inoculated intravenously with various doses of the same virus stock. Three weeks later, serum from each animal was tested for the presence of RLV antigens by immunoblotting.

Table 2

Immune protection following low-dose inoculation with live, pathogenic RLV

	Group	Initial RLV Dose (AID_{50}) [a]	Fraction Virus Free	RLV challenge (AID_{50}) [b]	Fraction Virus Free
Expt 1	1	2	3/10	200	2/3
	2	0.4	7/10	200	2/7
Expt 2	1	2	3/10	20	1/3
	2	0.4	7/10	20	1/7

a - Animals (10 mice per group) were inoculated iv with the indicated amounts of RLV on day 0 and serum tested on day 21 for RLV antigens by immunoblot analysis and XC-assay. Only animals which received initial doses that were 100% infectious in nude mice, and therefore, contained definate live virus are shown.

b - Mice shown to be virus free following initial inoculations were challenged with acute doses of RLV and serum was reanalysed for RLV antigens 21 days afterwards.